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Micro-Pharmacokinetics by Finger-Prick: Acetaminophen

John V Schloss*

Marshall University, USA

***Corresponding Author:** John V Schloss, Marshall University, One John Marshall Drive, Huntington, West Virginia 25755, USA.

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Abstract

A facile method for sample collection and work-up has been developed that enables rapid analysis of blood drug levels. After collecting a small volume of blood (5 µL) by use of a lancing device, the sample is diluted with methanol containing 25 mM ammonium formate (195 µL) in an auto sampler vial micro-insert. After mixing the sample and allowing precipitated material to settle out, a small volume of the supernatant (50 µL) is transferred to a clean micro-insert in a second auto sampler vial. The extracted sample is suitable for direct analysis by tandem mass spectrometry. By use of this method, acetaminophen can be detected by finger-prick within one minute after oral administration of a single 500 mg tablet. A complete set of pharmacokinetic parameters were obtained for an individual subject by use of the micro-pharmacokinetic finger-prick method and compared to literature values determined by more conventional methods, demonstrating the method's suitability for acetaminophen and potential use for a wide range of drug substances.

Keywords: *Acetaminophen; Micro-pharmacokinetics; Finger-prick; Mass spectrometry; Individualized-medicine*

Abbreviations: APAP: Acetaminophen; LC-MS/MS: Liquid Chromatography interfaced with tandem mass spectrometry; GSH: Glutathione; NAPQI: *N*-acetyl-p-Benzoquinone imine; SRM: Single Reactant Monitoring; MRM: Multiple Reactant Monitoring; m/z: Mass to charge ratio; *Cmax*: Maximum concentration of drug achieved in the blood; *Tmax*: Time at which the maximum concentration of drug is achieved in the blood; *AUC*: the area under the curve for blood drug levels in a pharmacokinetic profile; LOQ: Limit of Quantitation.

Introduction

Modern tandem mass spectrometers have dramatically reduced the time and amount of sample required to define drug pharmacokinetics. Blood samples obtained by finger-prick are more than adequate to accurately measure the blood-levels of virtually all drugs, even those administered in small doses. To take full advantage of the advances in analytical instrumentation, there is a need to develop simple protocols for sample collection and sample work-up that reduce the amount of time, cost, and effort devoted to sample preparation. Dried blood spot analysis has proven to be amenable to automation and is substantially more convenient than more traditional methods of sample work-up [1]. However, dried blood spot analysis still requires extraction of the analyte before analysis; introduces inaccuracies related to the amount of sample actually analyzed; and exposes the analyte to conditions during the drying process that may further confound the analysis. To further simplify the process for analyzing exceedingly small blood samples, a direct micro-precipitation protocol has been devised. This method employs supplies and equipment commonly found in most mass spectrometry laboratories.

For less-sensitive analytical methods, solid-phase extraction is most commonly employed for sample preparation. Solid-phase extraction can handle much larger volumes of biological samples and can be used to concentrate analytes during elution from the solid support. Despite new developments in sorbants for analyte extraction and methods for micro-extraction, solid-phase extraction still has greater potential for analyte loss; requires more effort in developing the sample-handling protocol; and has more need for method validation by use of isotopically-labeled internal standards [2-3].

Methanol is a commonly used precipitant to eliminate macromolecules prior to subjecting samples to analysis by tandem mass spectrometry [4]. Inclusion of a volatile salt, such as ammonium acetate or ammonium formate, in the precipitant enhances separation of low molecular weight analytes from macromolecules and provides a simple, one-step method for sample handling that produces an extract suitable for direct analysis [5]. However, blood samples are usually collected by venipuncture and held for an unspecified period of time before being subjected to analyte extraction. During the process of blood collection and storage, there is potential for loss of the analyte due to degradation or adsorption onto the surfaces of collection vials. To minimize the time for analyte degradation and sample loss through handling, a method has been developed for immediate work-up of small blood samples, obtained by finger-prick, with ammonium formate-methanol. This process can be conducted on a small scale by use of an auto sampler vial insert. Use of glass inserts and autosampler vial caps lined with PTFE further reduce the chances for analyte loss due to adsorption prior to conducting analyses. The vast majority of low molecular weight drug substances and biomarkers should be compatible with this sample preparation method for analysis by LC-MS/MS.

To demonstrate the utility of the micro-pharmacokinetic method, acetaminophen was chosen as a representative drug substance. Since acetaminophen was first synthesized by Harmon Northrop Morse in 1873 [6], there have been concerns about its toxicity. Acetaminophen did not become a commercial product in the United States until 1955, when it was sold under the brand name Tylenol by McNeil Laboratories. In 1956 acetaminophen, also known as paracetamol, was first sold in the United Kingdom by a subsidiary of Sterling Drug as Panadol. Acetaminophen, known by the abbreviation APAP in the United States, is the active metabolite of both acetanilide and phenacetin, which preceded APAP as commercial products in clinical use. Acetanilide was linked to the development of methemoglobinemia by Bernard Brodie and Julius Axelrod in 1948, which led to its replacement by APAP [7-8].

APAP can both stimulate and inhibit the activity of prostaglandin H synthase at low and high concentrations, respectively [9]. Prostaglandin synthase acts on APAP as a substrate to transform it by one- and two-electron oxidations into *N*-acetyl-p-benzoquinone imine (NAPQI); the dimer 4'4'''-dihydroxy-3', 3'''-biacetanilide (bi-APAP); and other polymeric products. NAPQI is a toxic oxidation product of APAP that is also formed in much higher quantities by cytochrome P450 enzymes, primarily CYP3A4 at therapeutic concentrations (50 μM) and also by CYP2E1, CYP1A2, and CYP2D6 at higher, toxic concentrations (1 mM) [10]. Depletion of glutathione (GSH) by NAPQI is thought to be the primary cause of hepatic injury and deaths, due to liver failure, from APAP overdose [11]. At higher concentrations of APAP, the normal mechanisms for elimination by glucuronidation and sulfation become saturated, allowing for its oxidation to NAPQI.

APAP has been reported to be somewhat selective (four-fold) for the inducible prostaglandin synthase isozyme COX-2 [12]. Selective inhibition of the brain-specific isozyme COX-3 was offered as an explanation for the CNS-specific pharmacology of APAP [13]. However, this suggestion has been largely discredited, since humans do not express a functional form of this isozyme [14-15]. Potent inhibition of COX-2 by APAP appears to require cellular integrity, since the enzyme is inhibited at therapeutic levels in intact cells, but not in broken cells [16]. This observation can be explained by the effect of 'peroxide tone' on COX-2 inhibition by APAP; the fact that hydroperoxide-generating lipoxygenases lessen the potency of APAP; and the buildup of COX substrates in the periphery to minimize the effectiveness of APAP [17]. The molecular mechanism of APAP appears to involve quenching of an essential radical at tyrosine-385 in the COX active site. The potency of APAP depends on failure to regenerate this tyrosyl radical, which explains its lack of potency in the periphery, where radical regeneration occurs more rapidly. The pharmacology of APAP may also involve, in part, its metabolites, such as the phosphodiesterase/transacylase-dependent AM404, which binds to the cannabinoid receptor, or GSH conjugates (APAP-GSH), which may be responsible for mitigating D-serine-induced oxidative stress [18]. The hepatic toxicity of APAP is likely exacerbated by its inhibition of COX-2, since COX-2 prevents hepatotoxicity by 'death proteins', such as calpain, phospholipases, and nucleases. APAP is a leading cause of acute liver failure and has been associated with neurodevelopmental and behavioral disorders when taken during pregnancy [18].

Given the complex pharmacology of APAP and its hepatotoxicity associated with GSH depletion by the NAPQI metabolite, a rapid, less-invasive method for concurrently monitoring APAP and GSH has clinical utility. A rapid method for analyzing blood levels of APAP, other drugs, and biomarkers for drug toxicity has been employed here to follow APAP and GSH levels in blood samples obtained by finger-prick.

Materials and Methods

Sample Preparation

Blood samples were collected from a single 63 year-old male subject before and after administering a 500 mg APAP tablet, following an overnight fast. An Accu-Chek® FastClix® (Roche) lancing device was employed to obtain 5 µL of blood by finger-prick. The blood sample was then quantitatively transferred to an autosampler vial insert (Thermo Scientific/National Scientific C4012-465; contained in a 12 x 32 mm, 2 mL, 8-425 screw thread vial; sealed with a polypropylene cap and PTFE/silicone septum) containing 195 µL of 25 mM ammonium formate in methanol by use of a 2-20 µL Fisher brand Elite micropipette. After thoroughly mixing each sample and allowing the precipitated blood proteins to settle out, one 50-µL aliquot was transferred to a fresh 200-µL auto sampler vial insert and a second 10-µL aliquot was separately diluted with 0.99 mL of water in an auto sampler vial. The 1/40 (methanol) and 1/4000 (water) blood-sample dilutions were then analyzed on an Agilent 6490 Triple Quadrupole LC/MS.

LC-MS/MS

An Agilent 6490 Triple Quadrupole LC/MS equipped with an Agilent Infinity 1260/90 liquid chromatography system was employed for analysis of extracted blood samples. Chromatography was conducted by use of an Agilent Poroshell 120 EC-C18 column operated at 40°C and eluted with a linear gradient of water-methanol (5-100%, 10 min); methanol (100%, 5 min); and 5% methanol in water (5 min) at 0.4 mL/min. All solvents employed in chromatography contain 25 mM ammonium formate to enhance sensitivity and suppress formation of sodium adducts. Drugs and drug metabolites were detected by use of an Agilent 6490 Triple Quadrupole mass spectrometer set up to monitor 144 different channels for SRM or MRM protocols. In this system, APAP can be observed as a discrete peak running at 2.84 min (152→110 m/z) in the chromatographic profile.

Results and Discussion

Figure 1 displays a 10-min chromatographic run, with a 10-min column regeneration cycle, separating the Agilent Comprehensive Forensic Toxicology test mixture to which APAP has been added. In Figure 1, 144 possible channels, corresponding to different drugs and drug metabolites in the Agilent Comprehensive Forensic Toxicology mixture, are displayed. All compounds are present in aqueous solution at concentrations of 100 ng/mL. APAP is visible, in the profiles displayed in Figures 1 and 2, as a peak eluting at 2.84 min. All of the other drugs and drug metabolites, present in the mixture, elute at later times in this chromatographic profile, with similar, higher, or lower sensitivity than APAP.

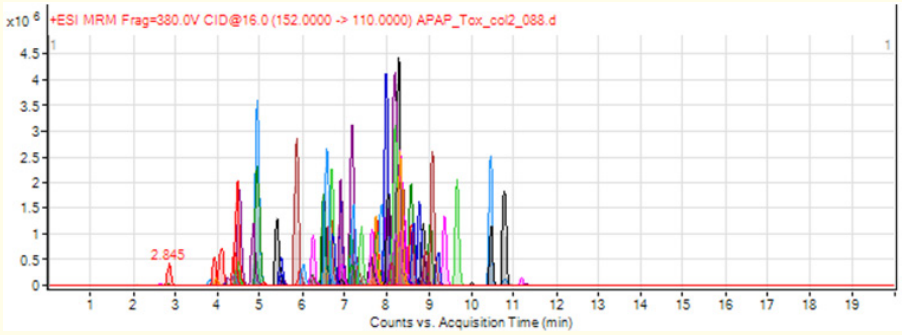

Figure 1: *A chromatographic profile for a mixture of APAP and 108 other drugs.*

Figure 2 displays the same chromatographic profile displayed in Figure 1, for which only the channel specific to APAP is shown and the other 144 channels, specific to other drugs in the Agilent Comprehensive Forensic Toxicology mixture, are turned off. In Figure 2, only the peak specific to APAP can be observed and there is no interference from any of the other drugs or metabolites present in the mixture. This result illustrates the discrimination and selectivity obtained by tandem mass spectrometry that enables selective detection of APAP, or other drug, in a complex biological matrix, such as extracted human blood.

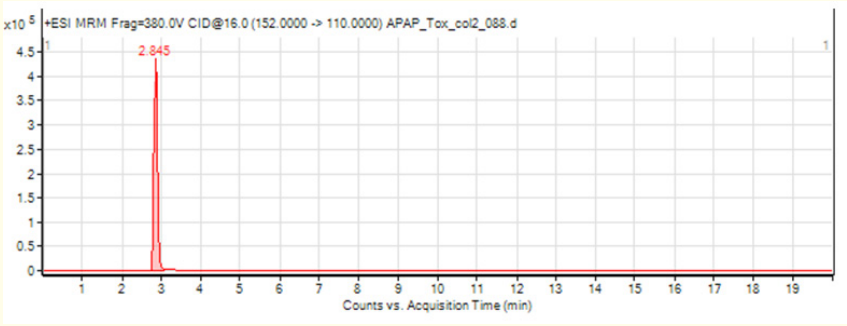

Figure 2: *The same profile shown in Figure 1 with only the APAP channel displayed.*

A standard curve for APAP is illustrated in Figure 3. The mass spectrometer gives a linear response for a 5 µL injection of 0.050 ng/mL (0.33 nM) APAP up to a concentration of 500 ng/mL, (3.3 µM), but becomes non-linear at higher concentrations. For this system, the limit of quantitation (LOQ) for APAP is well below 0.05 ng/mL (0.33 nM). One 500-mgAPAP tablet, fully dispersed in an average adult, would give a drug concentration more than 50,000-times the limit of detection for the Agilent 6490 Triple Quadrupole LC/MS.

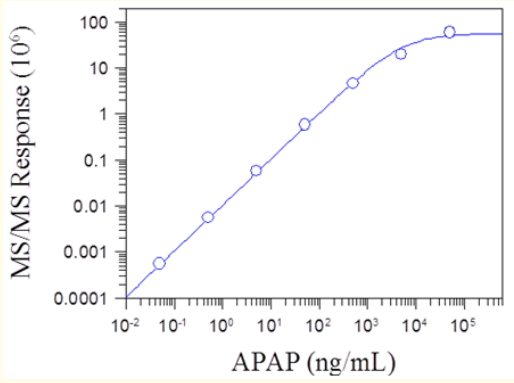

Figure 3: *A standard curve for APAP.*

Figure 4 illustrates the blood levels of APAP obtained, following administration of one 500-mg tablet of APAP. Prior to administration, APAP could not be detected in blood samples (< 2 ng/mL). Within 1 min following ingestion of a 500-mg APAP tablet, 120 ng/mL (0.8 µM) of APAP could be detected in a blood sample obtained by finger-prick. Maximum blood levels were observed at about 20 min (*Tmax*), following APAP ingestion, with a maximum concentration (*Cmax*) of 21.3 µg/mL (141 µM) achieved at that time (Table 1).

These values are similar to results obtained by finger-prick in a more limited study, involving only three sample collection times, 15, 30, or 60 min, after oral administration of one gram of APAP [19]. In that study [19], the *Cmax* and *Tmax* values obtained by finger-prick were > 22.9 µg/mL and < 15 min, respectively, compared to values of > 11.8 µg/mL and > 60 min for samples collected at the same time points by venipuncture. Typical values for *Cmax* and *Tmax*, after oral administration of one gram of acetaminophen and sample collection by venipuncture, are 8-16 µg/mL and 60-120 min, respectively [20-24]. The results presented in Table 1 are consistent with drug levels achieving a higher level (*Cmax*) at an earlier time (*Tmax*) in blood samples collected by finger-prick, than samples collected by venipuncture.

Time (min)	Concentration (μM)	Time (min)	Concentration (μM)
0	0.00	240	54.3
1	0.80	300	29.9
5	5.52	429	19.7
10	57.3	674	9.50
20	141	674	9.22
42	116	1162	2.94
60	82.1	1162	3.01
80	78.9	1561	1.02
122	69.5	1561	1.28
181	47.4	1561	1.37

Table 1: *APAP blood concentrations following oral administration of one 500 mg tablet.*

When data from Table 1 are presented in a linear-linear plot format (Figure 4), they more resemble bolus delivery of APAP by intravenous injection, than oral administration [22]. Fit of a single exponential (GraFit Data Analysis Software, Erithacus Software Limited) to the data collected more than 20 min after oral administration of APAP (42-1561 min) gave a zero-time intercept of 18 ± 2 μg/mL (120 μM) and an elimination rate constant of 0.34 ± 0.05 h^{-1} (half-time = 2.0 h). When the same data are presented in a log-log plot format (Figure 5), it is apparent that the elimination rate is biphasic with a rate constant of approximately 0.1 h^{-1} (half-time = 5 h; < 27%) for the slower, second phase. The *AUC* calculated from a single exponential fit gave a value, 53 h.μg/mL, that was comparable to other literature values (29-54 h.μg.mL^{-1} for one gram of orally administered APAP) [20-24]. The single exponential fit of data gives a reasonable determination of *AUC*, since estimates corrected for the true amount of APAP present in blood prior to 20 min or during the second phase of elimination (e.g., after 100 min) made little difference in the calculated value (< 15% change). Use of data analysis software that fit a one or two compartment model to pharmacokinetic data (e.g., Phoenix WinNonlin) tended to underestimate *AUC* and *Cmax*, possibly due to the unusual and rapid uptake kinetics of APAP.

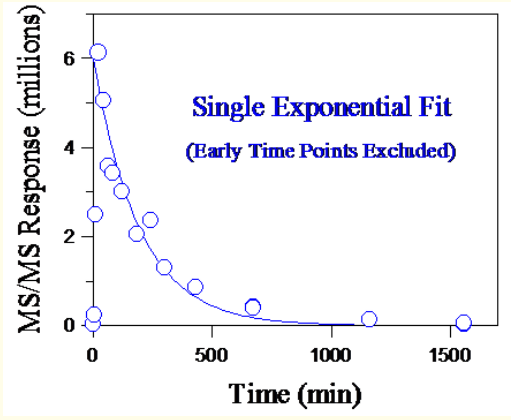

Figure 4: *Single exponential fit of APAP data.*

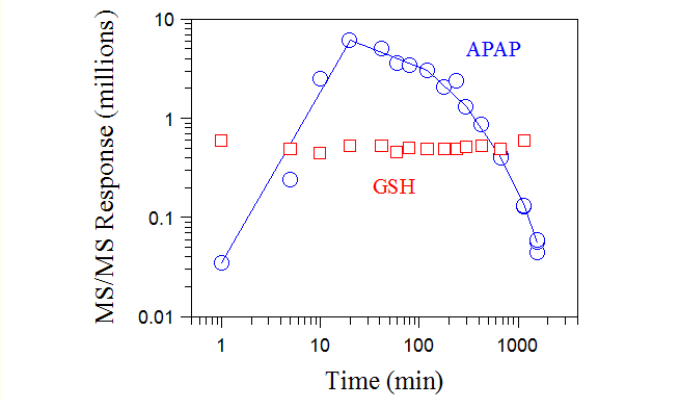

Figure 5: *APAP (⊙) and GSH (⊡) in blood samples, log-log format.*

Figure 5 also illustrates the blood GSH levels observed in samples collected at various time-points. Blood GSH can easily be determined concurrently with APAP analysis, since, as shown in Figure 1, the Agilent 6490 Triple Quadrupole LC/MS can monitor multiple compounds (up to 500 different ions) in the same chromatographic profile. There are no significant changes in blood GSH levels following oral administration of a single 500 mg APAP tablet. Since GSH is thought to protect against APAP hepatotoxicity and GSH depletion is associated with APAP toxicity [24], the ability to monitor GSH levels concurrently with APAP blood levels should be of special relevance to any individual's ability to tolerate APAP therapy.

Conclusion

The ammonium formate-methanol extraction procedure described here provides good separation of macromolecules from low molecular weight constituents present in human blood. A clear supernatant is obtained after mixing the precipitated blood extract by inversion at room temperature, without a need to subject the sample to centrifugation. However, the precipitated proteins and other methanol-insoluble macromolecules could be concentrated by centrifugation of the autosampler vial-microinsert container; the pellet washed by use of the ammonium formate-methanol solution and repeated centrifugation; and the resulting pellet, once freed of methanol-soluble analytes, subjected to further analysis (e.g., proteomics). APAP is obtained in the supernatant (0.8 μM/20 = 40 nM) within one minute after oral administration of a single 500-mg tablet. By analogy, this protocol should prove viable for almost all drug substances.

The blood APAP levels measured by use of the micro-pharmacokinetic finger-prick method described here compare favorably with expectations based on reported pharmacokinetic parameters. When monitored by finger-prick, the time (*Tmax*) required to achieve a maximum APAP blood level (*Cmax*) occurs earlier than when the blood samples are collected by venipuncture. The *Tmax* observed in the current study, 20 min, is comparable to a reported value, ~15 min [19], for data that was also based on blood obtained by finger-prick, but is much earlier than typical values for blood samples collected by venipuncture, 60-120 min [20-24]. The *Cmax* in the current study, 21 μg/mL, is also comparable to a reported value for blood samples collected by finger-prick, 23 μg/mL [19], and somewhat higher than values for samples collected by venipuncture, 8-16 μg/mL [20-24]. The area under the curve (*AUC*) obtained in the current study is within the range of values reported for samples collected by venipuncture, 29-54 $h.mg.mL^{-1}$. Overall, based on APAP, the pharmacokinetic data for blood samples collected by finger-prick will be similar to data derived from blood obtained by venipuncture, with the exception that drug progress curves will be shifted to earlier times.

Of particular importance to APAP pharmacology is the concurrent measurement of GSH levels. The level of GSH obtained in ammonium formate-methanol supernatants does not change across the entire pharmacokinetic profile for APAP, indicating that the 500 mg oral dose administered was not sufficient to cause toxicity. The ease of obtaining small blood samples and working each one up rapidly and individually, is an advantage of the micro-pharmacokinetic finger-prick method for APAP, especially if GSH levels are concurrently used as a biomarker for drug toxicity [11]. Many drugs can be detected with even greater sensitivity than APAP and should be suitable candidates for micro-pharmacokinetic analysis by finger-prick. The protocol described here should facilitate advancement of individualized medicine by enabling less invasive pharmacokinetic profiles with concurrent assessment of biomarkers for efficacy or drug-induced toxicity.

Conflict of Interest

There are no conflicts of interest.

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