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*Short Communication*

# Determination of antipyrine in human serum by direct injection restricted access media liquid chromatography

B.J. GURLEY,* S. ZERMATTEN and D. SKELTON†

*Department of Pharmaceutics, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*
† *Department of Pharmacy Practice, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*

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## Introduction

Antipyrine (AP) has been utilized extensively as a biochemical probe for studying hepatic oxidative metabolism in humans and animals [1–4]. More specifically, AP clearance from serum or plasma has been used as a determinant of hepatic cytochrome P-450 enzyme activity [5, 6]. By monitoring changes in AP clearance, the effect of various drugs and disease states on the activity of hepatic P-450 enzymes has been described [7–10]. Typically, AP clearance has been determined from the area under serum concentration–time profiles that were quantitated by liquid chromatography (LC) or capillary electrophoresis (CE). Previously reported chromatographic [11–14] and electrophoretic [15] methods for measuring AP serum concentrations require some degree of sample pretreatment (e.g. solvent extraction or protein precipitation) prior to analysis. In fact, the majority of drugs analysed in biological media by reversed-phase LC, AP notwithstanding, require some measure of sample preparation. Apart from solvent extraction and protein precipitation, other preparative measures include ultrafiltration, the utilization of disposable extraction columns and column switching devices [16]. From a clinical pharmacokinetic standpoint, excessive sample preparation can prolong the analysis time and subsequent turnaround time for drugs that require therapeutic monitoring. Consequently, any technology that reduces or eliminates the need for sample pretreatment will shorten analysis time and, in turn, improve sample throughput. The recent development of restricted access media (RAM) LC appears to be just such a technology [17].

Unlike conventional reversed-phase LC, restricted access media LC permits the direct injection of drug-containing serum [18–20], plasma [21] or whole blood [22] without damage to the analytical column. This is accomplished with a novel stationary phase that combines aspects of exclusion and reversed-phase chromatography [23, 24]. The column packing permits small drug molecules to interact with the reversed-phase moiety while restricting the access of large protein molecules. In short, RAM's unique mode of separation precludes the need for sample pretreatment called for in many LC assays. In this report we describe an RAM LC method for the determination of antipyrine in human serum. This method was used to construct an AP concentration–time profile in a healthy volunteer.

* Author to whom correspondence should be addressed.

## Experimental

*Materials*

Antipyrine, acetaminophen (internal standard), 4-hydroxyantipyrine, norantipyrine, glacial acetic acid, monobasic and dibasic potassium phosphate were purchased from the Sigma Chemical Co. (St Louis, MO, USA). LC grade acetonitrile, methanol and tetrahydrofuran were purchased from Burdick and Jackson Chemical Company (Muskegon, MI, USA). Deionized water was obtained from a Milli-Q plus ultra pure water system purchased from Millipore Corporation (Bedford, MA, USA). Drug-free human serum was obtained with informed consent from healthy volunteers.

*Instrumentation and chromatographic conditions*

A component LC system (Shimadzu Scientific Instruments, Columbia, MD, USA) consisted of a LC-600 solvent delivery system, a Model SIL-9A autoinjector, and a Model SPD-6A UV absorbance detector operated at 244 nm. A prepacked 25 cm × 4.6 mm i.d. semipermeable surface-C8 (SPS-5PM-5S-100, Regis Chemical Co., Morton Grove, IL, USA) LC column and guard column was operated with a mobile phase consisting of monobasic and dibasic potassium phosphate buffer (0.1 M, pH 7.4), acetonitrile and tetrahydrofuran (99:0.5:0.5, v/v/v). To improve antipyrine and acetaminophen peak symmetry, glacial acetic acid was added to the mobile phase to achieve a concentration of 0.01 M. Prior to use, each batch of mobile phase was filtered through a Duraporé 0.45-μm filter (Millipore, Bedford, MA, USA) and sonicated under vacuum for 30 min. The mobile phase was continuously sparged with helium and delivered at a flow rate of 1.0 ml $min^{-1}$. Column temperature was maintained at 37°C with a Model CTO-6A column oven (Shimadzu, Columbia, MD, USA). Detector output was recorded and chromatograms analysed by a C-R5A Chromatopac recorder/integrator (Shimadzu, Columbia, MD, USA).

*Standard solutions and sample prepartion*

Standards of AP and the internal standard acetaminophen (APAP) were prepared in drug free serum. Initial stock solutions of 1 mg $ml^{-1}$ were prepared in methanol for each compound. AP spiking solutions of 10 and 20 μg $ml^{-1}$, and an APAP spiking solution of 10 μg $ml^{-1}$ were then prepared by further dilution of each stock with methanol. Methanolic stock solutions were stored at −70°C and proved to be stable for over 3 months. Aliquots of the AP spiking solutions and 500 μl of the APAP spiking solution were added to clean borosilicate glass conical tubes, and the organic solvent evaporated under nitrogen. Human serum was filtered through a 0.22-μm Millex-GV filter (Millipore, Bedford, MA, USA) and 1 ml was added to each conical tube to give serum standards of 0.25, 1, 5 and 20 μg $ml^{-1}$. Each solution was mixed with a vortex mixer for 30 s and the serum transferred to a disposable 1.5 ml polypropylene microcentrifuge tube (Brinkman, Westbury, NY, USA). Using an Eppendorf Model 5414 microcentrifuge (Brinkman, Westbury, NY, USA), tubes were centrifuged at 12,000 rpm for 5 min. Serum aliquots were then transferred to autoinjector vials and a 25-μl volume was injected onto the SPS-C8 column. Fresh serum standards were prepared for every run.

Methanolic stock solutions of two principal AP metabolites (4-hydroxyantipyrine, and norantipyrine) were diluted in mobile phase to a concentration of 20 μg $ml^{-1}$ and 25-μl aliquots of each were injected.

*Treatment of clinical samples*

A healthy male volunteer, weighing 100 kg, ingested a 10 mg $kg^{-1}$ dose of AP as an oral solution. Serial blood samples (5 ml) were obtained by venipuncture over 48 h. Blood, collected in anticoagulant-free Vacutainer tubes, was allowed to clot and the serum separated by centrifugation. Serum samples were stored at −70°C until analysis. Upon analysis, serum samples were thawed and 1 ml was added to clean borosilicate glass conical tubes containing 5 μg of APAP. Samples were then mixed with a vortex mixer, centrifuged, and injected as described for serum standards.

*Method validation*

The absolute recovery of AP was calculated by dividing the slope of the calibration standards in serum by the slope of the standards in mobile phase. Peak area ratios (AP/APAP) were used for quantitative computations. Calibration curves were calculated by 'weighted' (1/conc.) least squares linear regression analysis using a commercial software package (DeltaGraph®, Monterey, CA,

USA). Accuracy and precision of the method were determined by replicate analysis of six known concentrations equally divided over the calibration curve. Inter- and intra-day accuracy was expressed as percentage deviation from the spiked value using the following equation.

$$\% \text{ Error} = \frac{(C_{\text{mean obs}} - C_{\text{spiked}})}{C_{\text{spiked}}} \cdot 100 \quad (1)$$

where $C_{\text{mean obs}}$ is the mean observed concentration for each standard and $C_{\text{spiked}}$ is the theoretical spiked concentration. The lower limit of quantitation was defined as the concentration of the lowest standard in the analytical run which was quantitated with a definite level of certainty (precision <10%).

## Results and Discussion

Typical chromatograms of blank human serum and serum spiked with 5 and 0.5 μg $ml^{-1}$ are shown in Fig. 1. Figure 2 depicts chromatograms from a pharmacokinetic study for serum samples obtained at 0 and 12 h after AP dosing. Retention times of AP and APAP were approximately 8.5 and 11.6 min, respectively. No interference from endogenous serum components, 4-hydroxyantipyrine, or norantipyrine was observed for AP or APAP. Although 4-hydroxyantipyrine and norantipyrine standards gave retention times of 16 and 21 min, respectively, these metabolites were not detected in human serum. This finding confirmed the results of previous studies which found that elimination rates of AP metabolites exceeded their rates of formation [25, 26]. The following drugs were also found not to interfere with the chromatography of AP and APAP: adenosine, aminopyrine, amoxicillin, atropine, butorphanol, gentamicin, indomethacin, pentobarbital and phenacetin.

Absolute recovery of AP was 99.7 ± 0.82% ($n$ = 4) over the whole concentration range. The method was shown to be linear over the AP concentration range of 0.25–20 μg $ml^{-1}$ in human serum, with a mean slope of 0.2875 (SD = 0.0079, $n$ = 10) and a mean intercept of −0.0197 (SD = 0.0093, $n$ = 10). Weighted linear regression analysis of AP/APAP peak area ratios vs spiked AP serum concentration gave a correlation coefficient of 0.9995 (range = 0.9990–0.9998, $n$ = 10). Intra-day and inter-day accuracy and precision of the method are presented in Tables 1 and 2. Data

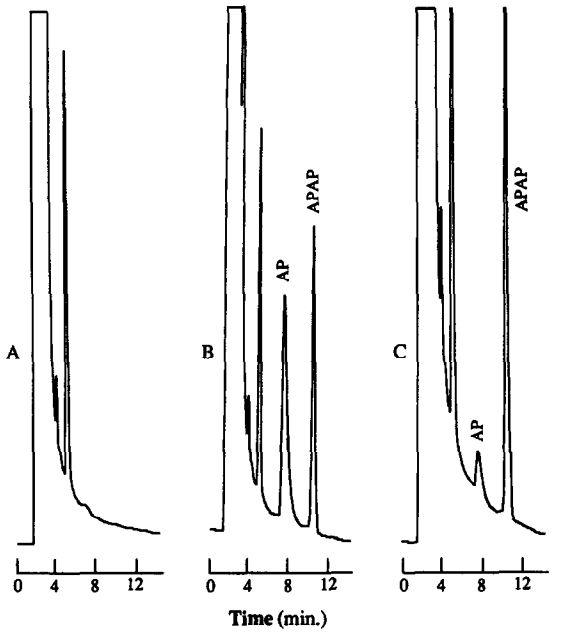


**Figure 1**
Typical chromatograms using the described method. (A) Blank human serum (AUFS = 0.04). (B) Human serum spiked with 5 μg $ml^{-1}$ AP and 5 μg $ml^{-1}$ APAP (AUFS = 0.04). (C) Human serum spiked with 0.5 μg $ml^{-1}$ AP and 5 μg $ml^{-1}$ APAP (AUFS = 0.01). For chromatographic conditions see Experimental section. AP = antipyrine, APAP = acetaminophen, AUFS = absorbance units full scale.

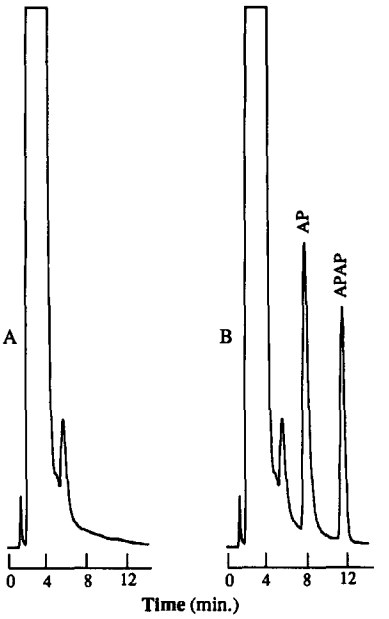


**Figure 2**
Chromatograms of AP-containing serum samples obtained from a human volunteer at 0 h (A) and 12 h (B) following ingestion of a 10 mg $kg^{-1}$ dose (AUFS = 0.04). For chromatographic conditions see Experimental section. AP = antipyrine, APAP = acetaminophen, AUFS = absorbance units full scale.

**Table 1**
Intra-day accuracy and precision for the analysis of antipyrine in human serum

| Concentration added ($\mu g\ ml^{-1}$) | Concentration found* ($\mu g\ ml^{-1}$) | RSD (%) | Error (%) |
|---|---|---|---|
| 20.07 | 20.04 ± 0.220 | 1.1 | −0.2 |
| 10.04 | 9.98 ± 0.240 | 2.4 | −0.6 |
| 5.02 | 5.01 ± 0.091 | 1.8 | −0.2 |
| 2.01 | 2.07 ± 0.052 | 2.5 | 3.0 |
| 1.00 | 1.04 ± 0.016 | 1.5 | 4.0 |
| 0.50 | 0.51 ± 0.016 | 3.2 | 2.0 |
| 0.25 | 0.252 ± 0.013 | 5.2 | 0.8 |

*Mean ± standard deviation based on $n = 6$.

**Table 2**
Inter-day accuracy and precision for the analysis of antipyrine in human serum

| Concentration added ($\mu g\ ml^{-1}$) | Concentration found* ($\mu g\ ml^{-1}$) | RSD (%) | Error (%) |
|---|---|---|---|
| 20.07 | 20.01 ± 0.224 | 1.1 | −0.3 |
| 10.04 | 9.99 ± 0.141 | 1.4 | −0.5 |
| 5.02 | 5.01 ± 0.038 | 0.8 | −0.2 |
| 2.01 | 2.01 ± 0.056 | 2.8 | 0.0 |
| 1.00 | 1.02 ± 0.026 | 2.5 | 2.0 |
| 0.50 | 0.51 ± 0.031 | 6.1 | 2.0 |
| 0.25 | 0.254 ± 0.026 | 9.2 | 1.6 |

*Mean ± standard deviation based on $n = 6$.

from both tables indicate that the method was both accurate and precise. In Table 2, the lowest standard (0.25 $\mu g\ ml^{-1}$) had a % RSD of 9.2 and a predicted error of 1.6%; this was taken as the limit of quantitation.

At a column temperature of 37°C and a flow rate of 1 ml $min^{-1}$, normal operating backpressure was approximately 100 kg $cm^{-2}$. Over 200 injections could be made before a significant increase in backpressure (≥120 kg $cm^{-2}$) and a subsequent loss of peak symmetry was observed. As a result, guard columns were routinely replaced after 200 injections.

The method developed herein was applied to the assay of serial AP concentrations in the serum of a human subject following a single dose. Figure 3 shows a concentration–time curve for AP in that subject. This novel assay should be useful in measuring serum AP levels for pharmacokinetic studies. Unlike all previously reported LC methods for determining AP in human serum or plasma, our direct injection RAM method required no sample prepartion prior to analysis. This advantage, coupled with a sample run time of less than 13 min, should facilitate turnaround times for laboratories involved in pharmacokinetic monitoring of AP.

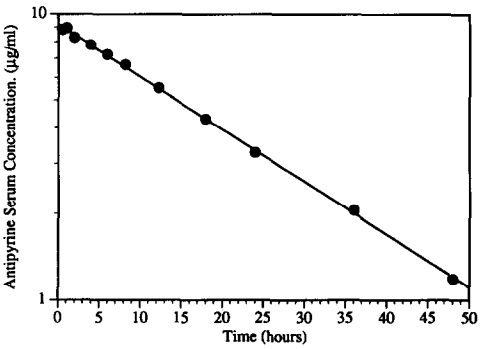


**Figure 3**
Concentration–time profile of antipyrine in a human volunteer as determined by direct serum injection RAM LC following ingestion of a 10 mg $kg^{-1}$ dose.

## Conclusion

A restricted access media LC method for the analysis of antipyrine (AP) in human serum has been described. With this method AP-containing serum aliquots could be directly injected onto the column without prior sample preparation. Endogenous serum components were eluted prior to AP and the internal standard. The assay was precise, with intra-day RSD ranging from 1.1 to 5.2%, and inter-day RSD ranging from 0.8 to 9.2%. Accuracy for

the method was excellent, with predicted errors less than 5%. The limit of quantitation was 0.25 μg $ml^{-1}$. A serum concentration–time profile from a healthy volunteer indicated that the method is applicable to AP pharmacokinetic studies.

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