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A validated method for the determination of paracetamol and its glucuronide and sulphate metabolites in the urine of HIV + /AIDS patients using wavelength-switching UV detection[1]

A. Di Girolamo [a,b], W.M. O'Neill [c], I.W. Wainer [d,*]

[a] *Département de Pharmacologie, Faculté de Médecine, Université de Montréal, Montréal, Quebec, Canada*
[b] *Abbott Laboratories Limited, St. Laurent, Quebec, Canada*
[c] *Divisions of Experimental Medicine and Medical Genetics, Department of Medicine, McGill University, Montreal, Quebec, Canada*
[d] *Department of Pharmacology, Georgetown University Medical Center, 3900 Reservoir Road NW, Room C-305 Medical-Dental Building, Washington, DC 20007-2197, USA*

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Abstract

Paracetamol is a safe drug which has been used as an in-vivo probe to determine phase II metabolism in a HIV + /AIDS population. Due to the biohazard nature of HIV-infected samples, a high performance liquid chromatography (HPLC) assay which offers minimal sample manipulation and maximal specificity was developed. This reverse-phase HPLC method uses wavelength-switching UV detection for the simultaneous determination of paracetamol and its glucuronide and sulfate metabolites in HIV-infected urine samples. The solvent systems involves a simple isocratic elution with a composition of 50 mM sodium acetate buffer, pH adjusted to 3.5; acetonitrile (96:4 v/v) modified with 0.35% trifluroacetic acid. The validated method is highly reproducible with an inter-assay variation of $< 7\%$. This method also shows good precision and sensitivity, making it an ideal assay for phenotyping studies to determine the extent of glucurondiation and sulfation activities.

Keywords: Paracetamol; Phase II metabolism; Glucuronidation; Sulfation; Phenotyping; HIV + /AIDS

1. Introduction

The intra-individual pharmacokinetics of many drugs vary considerably. These differences are largely due to differences in the metabolism, distribution and elimination of the therapeutic agents [1]. Metabolic differences are often the result of genetic polymorphism, drug interactions

* Corresponding author. Tel: + 1 202 6871650; fax: + 1 202 6875015; e-mail: waineri@gunit.georgetown.edu

[1] This manuscript is dedicated to Professor Anthony Fell in recognition of his contributions to the pharmaceutical and biomedical applications of multiple wavelength and diode array techniques.

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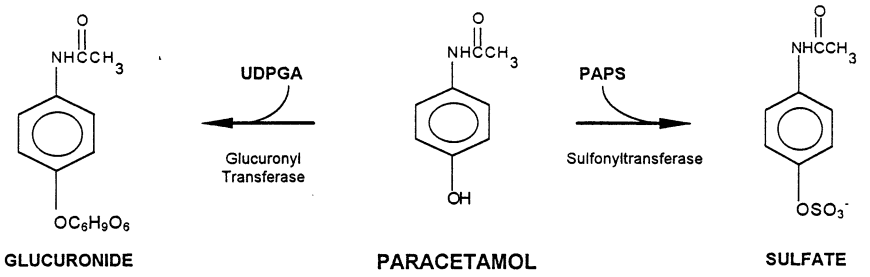

Fig. 1. Paracetamol and its primary metabolites: paracetamol-glucuronide and paracetamol-sulfate (conjugation of –OH group).

or environmental factors such as disease status, diet, lifestyle, etc. For enzymes which are genetically polymorphic, their activity falls into two clearly defined categories: individuals with reduced capability to metabolize certain drugs i.e. 'poor metabolizers' and those who demonstrate a regular pattern of metabolism i.e. 'extensive metabolizers'.

While genetic polymorphism is responsible for many inter-individual differences in response to drug therapies, much of the variability is due to non-genetically produced variations in the expression of these drug-metabolizing enzymes [2].

Changes in the relative levels and activities of metabolizing enzymes can be produced by drug interactions, environmental factors (i.e. smoking and alcohol intake), and clinical status such as disease progression [3] or malnutrition [4]. This implies that the expression of metabolic activity of a certain enzyme may change as a result of degeneration of a person's health. This phenomenon has been demonstrated in AIDS patients for the non-microsomal enzyme *N*-acetyltransferase II (NAT2) [5–7] and for the microsomal enzyme CYP2D6 [8], where patients with 'extensive metabolizer' genotypes expressed 'poor metabolizer' phenotypes as a result of disease status.

The genetic basis for polymorphic metabolism has been identified for a number of enzymes such CYP2D6 and NAT2 [7,8]. The functional activities of these enzymes as well as many others can be determined by using probe drugs such as caffeine (NAT2) or dextromethorphan (CYP2D6) [7,8]. In critically ill patients, probe-drug phenotyping can be a key element in designing clinical treatment. Paracetamol (APAP) is one such probe drug.

APAP is a widely used and relatively safe analgesic/antipyretic. When given in therapeutic doses it is metabolized primarily by conjugation to form glucuronide and sulfate derivatives (phase II metabolism) [9–12], Fig. 1. A small proportion is oxidized by the cytochrome P450 system to produce a highly reactive intermediate metabolite *N*-acetyl-*P*-benzoquinoneimine (NABQI) which is usually detoxified by conjugation with glutathione [9,13]. When glutathione stores are depleted, the formation of NABQI can result in liver injury [12,14].

APAP is an ideal drug for the study of factors influencing phase II drug metabolism in humans [15]. It is safe, user-friendly probe-drug which can be used to determine the phase II metabolism in HIV + /AIDS patients. The extent of glucuronidation is important in antiretroviral therapy since it is the primary pathway responsible for the metabolism of AZT, and, to a lesser extent, is involved in the metabolism of protease inhibitors. As treatment for HIV moves towards combination therapy of nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), AZT remains the cornerstone of this combination therapy. The addition of one or more PIs to the treatment regimen is quickly becoming the standard of care in HIV. Therefore, phase II metabolism plays an important role in the pharmacological and toxicological outcomes of these therapeutic agents in the HIV + /AIDS population.

Many high performance liquid chromatography (HPLC) methods exist for the assay of APAP and its metabolites in biological fluids [16–21]. However, many of these assays were developed for

toxicological purposes and used for an otherwise healthy population. These assays did not suit the requirements for more detailed studies of phase II metabolism in a very ill population exposed to polypharmacy. Our present studies involve the determination of APAP and its glucuronide and sulfate metabolites in multiple, HIV-infected urine samples. Due to the biohazard nature and multiple component matrix of the samples, the method required minimum sample manipulation and maximum specificity and sensitivity. The paper reports the development of a specific HPLC assay using wavelength-switching UV detection for the simultaneous determination of paracetamol and its glucuronide and sulfate metabolities in urine samples of HIV-infected patients. Results of validation including recovery, accuracy and precision, and sensitivity of this assay are reported.

2. Experimental

2.1. Chemicals

4-Acetamidopheol (paracetamol) and *p*-acetamidophenol-*β*-*d*-glucuronide (paracetamol-glucuronide) were purchased from Sigma (St. Louis, MO). Paracetamol-sulfate was a gift from MacNeil Consumer Products (Fort Washington, PA). HPLC-grade acetonitrile and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Trifluroacetic acid (TFA) was purchased from Sigma.

2.2. Standard stock solutions

Aqueous stock solutions of paracetamal (APAP), paracetamol-glucuronide (APAP-G) and paracetamol-sulfate (APAP-s) were each prepared in polypropylene tubes at concentration of 0.21, 8.45 and 5.00 mg ml^{-1}, respectively, and stored at -20°C.

2.3. Separation chromatography

2.3.1. HPLC system

The reverse-phase chromatographic system was composed of a Spectra Physics binary pump model P1500 and a Spectra Physics SP8875 autosampler equipped with a 20-μl sample loop, and a Spectra Physics UV1000 detector (Spectra-Physics, San Jose, CA). A Spectra Physics Datajet integrator connected to a Spectra 386 computer using the software winner on windows was used for electronic data collection.

Chromatographic separation of paracetamol and its metabolities was carried out with a Phenomenex ODS column 25 cm × 4.6 mm ID (Phenomenex, Torrance, CA) fitted with 5 micron C_{18} guard column (Regis, Morton Grove, IL).

2.3.2. Mobile phase

The compounds of interest were separated with a mobile phase composed of sodium acetate buffer (50 mM, pH 3.5): acetonitrile (96:4 v/v) modified with 0.35% TFA. At 25 min, the acetonitrile composition was increased to 25%; at 30 min the mobile phase was returned to the original composition and the column was re-equilibrated for 15 min.

2.3.3. UV detection

Detection of compounds was by UV, using a wavelength-switching program. The UV detector was programmed at 260 nm from 0 to 14 min the UV detector switched to 240 nm and autozeroed, and continued to detect at this wavelength until 25 min. At 25 min, the UV detector switched back to 260 nm. The detector was autozeroed at the beginning of each injection.

2.3.4. Chromatographic conditions

The chromatography was carried out using a flow rate of 1.0 ml min^{-1} at ambient temperature.

2.4. Probe-drug phenotyping method

One hundred and fifty subjects actively followed at the Montreal General Hospital (MGH) Immunodeficiency Treatment Centre (IDTC) and 36 seronegative controls were recruited into this study. IRB approval from the MGH ethics committee has been obtained prior to the start of the study, written informed consent was obtained

from each subject prior to the initiation of the phenotyping studies. Clinical data including a detailed HIV history, concomitant medications, past illness and current clinical status was obtained at time of enrolment. The participants were also given a questionnaire to complete as part of the phenotyping protocol.

Participants in this study were probe-drug phenotyped with a single oral dose of 500 mg of paracetamol (Atasol Forte®). A kit containing a 500 mg tablet of Atasol Forte®, and two urine specimen containers properly labelled were given to the participants along with instructions for dosing and storing of samples. Prior to the ingestion of the probe-drug, a pre-dose blank urine (20–40 ml) sample was obtained. Following the ingestion of the paracetamol, a 4-h spot urine (20–40 ml) was obtained. The urine samples were returned to Pharmacokinetics/Pharmacogenetics Laboratory at the Montreal General Hospital. The samples were pipetted into four polypyrene tubes (4 ml), each containing an aliquot of 3 ml of urine. The samples were heated at 60°C for 60 min providing for the inactivation of any virus and then stored at −20°C until analysis.

2.4.1. Sample preparation

After thawing, the urine samples were diluted 1:20 with deionized water to give a final volume of 1 ml. One hundred microliters of the sample were transferred to a 200 μl polypropylene autosampler vial and 20 μl was injected onto the HPLC column.

2.5. Standard curves

A 6-point standard curve was prepared by adding known concentrations of paracetamol and its metabolites, covering the ranges anticipated in the study to drug-free pooled and filtered urine. A 5 ml stock solution of spiked urine diluted 1:20 containing APAP: 50 μg ml^{-1}, APAP-G: 5000 μg ml^{-1} and APAG-S: 2000 μg ml^{-1} was prepared. This stock solution was serially diluted to obtain concentrations ranging from 5–50 μg ml^{-1} of APAP; 500–5000 μg ml^{-1} of APAG-G and 200–2000 μg ml^{-1} of APAP-S

2.6. Assay validation

For intra-day and inter-day validation studies, control samples were prepared from drug-free pooled and filtered urine spiked with paracetamol and its metabolites added at three different levels. The highest level of concentration contained 40.0 μg ml^{-1} of APAP, 4000.0 μg ml^{-1} of APAP-G and 1600.0 μg ml^{-1} of APAP-S. The medium level of concentration contained 30.0 μg ml^{-1} of APAP, 3000.0 μg ml^{-1} of APAP-G and 1200.0 μg ml^{-1} of APAP-S. The lowest level of concentration contained 5.5 μg ml^{-1} of APAP, 550.0 μg ml^{-1} of APAP-G and 220.0 μg ml^{-1} of APAP-S.

Recovery of the compounds of interest was tested in urine at the three different levels mentioned above. The control samples used in the validation were stored at −20°C and have been re-assayed over a 6 month period. There were no significant changes in the chromatographic results indicating that the samples are stable for at least this period of time.

3. Results and discussion

3.1. Chromatography

The paracetamol-glucuronide and paracetamol-sulfate were detected at 260 nm with retention times of 4.8 and 8.5 min, respectively. Paracetamol was detected at 240 nm with a retention time of 19 min. Under the chromatographic conditions utilized in this study, baseline separation of paracetamol and its metabolites was accomplished in biological samples.

The detection of APAP-G and APAP-S at a wavelength of 290 nm allows for the elimination of background noise with a minimal loss of sensitivity, since both these compounds are present in large concentrations. Switching to 240 nm to detect the APAP peak increases the sensitivity and allows for maximal UV absorbance of this peak which has the smallest urinary concentration and is present in only 1/100 of the concentration of the other two peaks.

No interfering peaks were found in the

pre-dose blank urine. Trace A in Fig. 2 is a chromatogram a pre-dose blank urine, trace B is a chromatogram of a spiked urine; and trace C is a chromatogram of a patient sample, 4 h post-dosing with 500 mg of APAP.

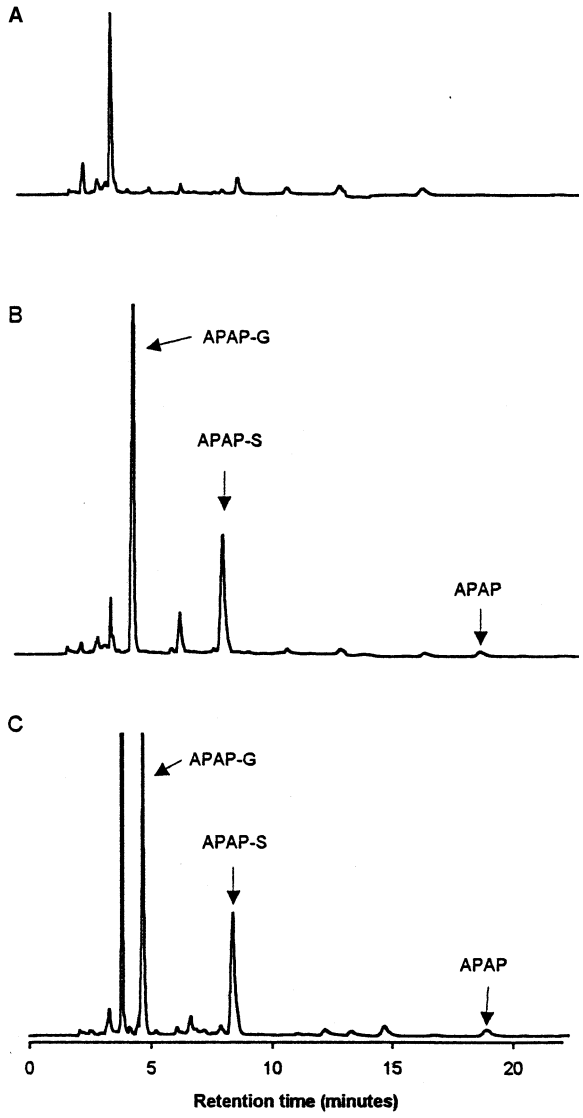

Fig. 2. (A) Chromatogram of blank urine. (B) Chromatogram of drug-free urine spiked with 40 μg ml^{-1} of APAP, 4000 μg ml^{-1} of APAP-G and 1600 μg ml^{-1} of APAP-S. (C) Chromatogram from a sample of a patient, 4 h post-dosing with APAP.

Table 1
Urinary recovery of APAP and its metabolites in human urine samples ($n = 5$)

Compound	Concentration (μg ml^{-1})	Recovery (%)	CV (%)
APAP	5.5	103.9	4.0
	30.0	108.2	5.2
	40.0	119.3	5.9
APAP-G	550	103.4	2.9
	3000	101.1	1.0
	4000.0	103.6	1.3
APAP-S	220.0	101.9	4.7
	1200.0	100.7	2.7
	1600.0	101.9	2.0

3.2. Validation

Recoveries of the compound of interest were calculated at three levels between the limits of quantification by comparing the peak areas of the concentrations from spiked urine samples with peak areas from spiked aqueous samples. The recoveries were $\geq 100\%$ for all compounds with a CV (coefficient of variation) of $< 6\%$. The results are tabulated in Table 1.

A linear correlation-response was found over a range of 5–50 μg ml^{-1} for APAP, 500–5000 μg ml^{-1} for APAG-G and 200–2000 μg ml^{-1} for APAG-S. The correlation coefficients (r) were in the range 0.9986–0.9999 while the regression coefficients (r^2) ranged from 0.9971 to 0.9997.

The results of the intra- and inter-day precision and accuracy are given in Table 2. In all cases, accuracy was within 13% of the theoretical for both intra- and inter-day variability. The precision calculated as the coefficient of variation (CV) was $< 5\%$ for intra-day variation and $< 7\%$ for inter-day variation for all compounds.

4. Conclusion

The validated method described in a relatively simple, sensitive and rapid assay which can be used in phenotyping studies to determine in-vivo, phase II metabolism. It is currently in use in a study of disease and drug effect on phase II

Table 2
Accuracy and precision of the analysis of APAP and its metabolites in human urine samples

Compound	Actual concentration (μg ml^{-1})	Measured concentration (mean ± SD)	Accuracy (%)	CV (%)
Intra-day validation ($n = 3$)				
APAP	5.5	6.2 ± 0.094	113.1	1.51
	30.0	31.4 ± 1.056	104.7	3.36
	40.0	42.9 ± 0.659	106.4	2.17
APAG-G	550.0	600.8 ± 25.859	109.2	4.30
	3000.0	2943.6 ± 45.325	98.1	1.54
	4000	4036.6 ± 58.479	100.9	1.44
APAP-S	220.0	249 ± 7.328	113.3	2.94
	1200.0	1235.2 ± 18.807	102.9	1.52
	1600.0	1654.4 ± 23.713	103.4	1.43
Inter-day validation ($n = 3$ per day × 4 days)				
APAP	5.5	6.2 ± 0.169	112.8	2.12
	30.0	31.9 ± 1.250	106.4	3.92
	40.0	43.6 ± 1.222	108.3	2.19
	550.0	604.1 ± 37.896	109.8	6.27
	3000.0	3033.0 ± 104.535	101.1	3.43
	4000.0	4112.3 ± 98.216	102.7	2.31
APAS-S	220.0	254 ± 8.663	115.4	3.4
	1200.0	1263.7 ± 40.085	105.3	3.2
	1600.0	1676.1 ± 39.730	104.3	2.0

metabolism in HIV + /AIDS patients. Urine samples from 150 HIV + /AIDS patients and 36 seronegative controls have been analyzed using this method. Results from this study will be reported elsewhere.

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