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Impact of Plasma Hemolysis on the Recovery of Phenprocoumon in LC-MS/MS Chiral Assay

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OVERVIEW

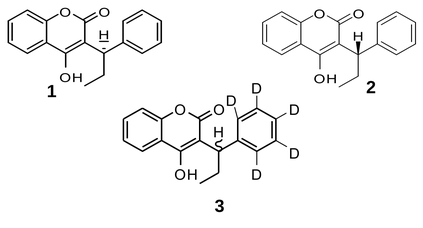
- **Novel Aspects**
  Overcoming a recovery loss in hemolyzed plasma samples by the addition of oxalic acid to the sample extraction.
- **Methods**
  Chiral chromatography: Astec Chirobiotic-V with mass spectrometry detection was performed on Agilent Technologies 1100 equipped with a triple quadrupole API 3000 (AB Sciex).
- **Results**
  Influence of some buffers and buffer systems containing iron ions and chelating agents such as EDTA and Oxalic acid was investigated.

INTRODUCTION

Hemolysis is defined as red blood cell breakdown and the release of hemoglobin and intracellular contents into the plasma. In bioanalysis, hemolyzed samples are often observed due to: in-vivo hemolysis related to pathological conditions, such as autoimmune hemolytic anemia or transfusion reaction, and in-vitro hemolysis due to improper specimen collection, specimen processing, or specimen transport.

During the method development for the quantification of phenprocoumon (**1**, **2**) in human plasma **(Figure 1)**, we observed strong recovery variation and loss (lower than 10% recovery) for hemolyzed plasma samples. The selected stable-isotope labeled internal standard phenprocoumon-d5 (**3**) tracks well the drug in which the ratio (drug/IS) remains the same independently of the recovery variation (loss of 90 % of the recovery) for artificially prepared hemolyzed QC samples.

Figure 1: Structures of:
(R)-Phenprocoumon (1), (S)-Phenprocoumon (2), (R,S)-Phenprocoumon-D5 Internal Standard (3)



METHOD

Stock solutions used were prepared in MeOH. Calibrants and QC samples were spiked in human plasma (K_3EDTA) for a range of 5.00 ng/mL – 1000.00 ng/mL. A liquid/liquid extraction procedure was used:

- Aliquot 200µL of plasma into tubes.
- Add 50µL of internal standard working solution (phenprocoumon-d5).
- Add 100 µL of buffer solution
  - ammonium bicarbonate
  - or, ammonium acetate
  - or, sodium carbonate
  - or, oxalic acid

Add 5 mL mixture of Chlorobutane:MTBE and vortex.

- Samples were then centrifuged and the organic layer was transferred and evaporated to dryness.
- The residue was reconstituted with 400 µL of ACN:H_2O 50:50% v/v and stored before injection at 4°C.

CHROMATOGRAPHY:

Chiral chromatography was achieved by an Agilent Technologies 1100 using an Astec Chirobiotic-V (100 mm x 4.6 mm, 5µm) with isocratic elution mobile phase (mixture of ammonium formate with adjusted pH and MeOH), at flow rate of 0.650 mL/min and column oven temperature set at 10ºC.

MASS SPECTROMETRY:

Mass spectrometry detection was performed on a triple quadrupole API 3000 (AB Sciex) instrument equipped with TurboIonSpray source. The analyte and the IS were detected in negative electrospray mode using multiple reaction monitoring (MRM) set at (279->250) for phenprocoumon and (284->255) for isotopically labeled internal standard phenprocoumon-d5.

The mass spectrometer settings were: Turbo ionspray gas:7500 cc/min; Scan type:-MRM; Auxiliary gas flow :14.00; Curtain gas (CUR):10.00; CAD gas:6.00; Ion Spray Voltage:-2500.00 V; Source temperature:450ºC.

RESULTS

For the extraction yield investigation, QC hemolyzed plasma samples were used and back-calculated against a plasma calibration curve. Although the precision and accuracy, performed by back calculation based on the drug/isotopically labeled internal standard ratio, was maintained, the recovery of phenprocoumon and its internal standard in hemolyzed plasma QCs was found to be lower than 10%.

Ten hemolyzed plasma lots from different donors were analyzed and low recovery was confirmed in eight of them. The results are summarized in **Table 1**. The extraction yield of (R)-phenprocoumon from hemolyzed plasma samples can drop significantly and vary in large range from 2.6 up to 50%.

Table 1: Summary of (R)-Phenprocoumon Percent Extraction Yield (PEY) Experiments Performed with Hemolyzed Plasma Samples from Different Donors

Samples	Peak area of (R)-Phenprocoumon (counts)	Area Ratio	IS Peak Area (counts)	Extraction Yield** (%)
Solution* (non extracted)	3109982.7	4.4	704484.0	N/AP
Plasma	2519086.3	4.4	573459.8	**81.0**
Hemolyzed plasma donor 1	316196.3	4.5	68877.9	**10.2**
Hemolyzed plasma donor 2	1023381.2	4.6	225095.9	**32.9**
Hemolyzed plasma donor 3	259622.8	4.7	54861.7	**8.3**
Hemolyzed plasma donor 4	78973.0	4.8	16450.9	**2.5**

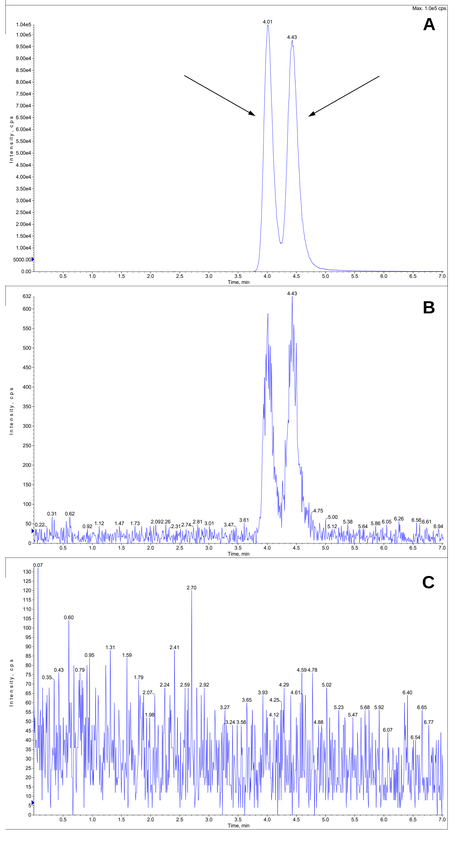
* This solution represents a theoreticaly calculated concentrations
**Percent Extraction Yield= Extracted Mean / Non-Extracted Mean x 100

Chiral chromatography showed that both enantiomers were affected by the same proportions so that the ratios between the enantiomers remained equivalent independently of the extraction yield. Representative chiral chromatograms of ULQ, LOQ and blank are presented in **Figure 2**. To investigate and to overcome this phenomenon, a series of experiments were performed.

The chelating ability of phenprocoumon at the extraction conditions was investigated by the addition of fero-/feri- ions to plasma. Possible reduction/oxidation or chelation in presence of fero-/feri- ions were also investigated. Addition of an excess of EDTA, a well known chelating agent, had also no improvement on the recovery. It was shown that the addition of hemoglobin and hemin to plasma had also no effect on the recovery. Additional experiments revealed that no drug was lost irreversibly due to the oxidation/reduction reactions in presence of hemoglobin, hemin, fero-/feri- ions.

In order to minimize hemolyzed plasma extraction yield variability, a systematical investigation of the influence of buffer on the percent extraction yield was performed. Different buffers such as ammonium bicarbonate, ammonium acetate, sodium carbonate, phosphate buffer, oxalic acid, ascorbic acid at different concentrations were tested.

Figure 2: Representative Chromatograms of Extracted ULQ (A), LLOQ (B) and Blank (C) Samples.



The analysis of the results revealed that the use of oxalic acid at concentration of 35 mM corrected the extraction yield of all hemolyzed plasma samples bringing it to the usual and acceptable rates of more than 85%. These new extraction conditions allow recovery of the analytes at rates of more than 85% for all plasma samples including the hemolyzed plasma samples **(Table 2)**.

Table 2: Summary of (R)-Phenprocoumon PEY Experiments Performed with Hemolyzed Plasma Samples Using Improved Extraction Procedure (35 mM Oxalic Acid)

Samples	Peak area of (R)-Phenprocoumon (counts)**	Area Ratio	IS Peak Area (counts)**	Extraction Yield*** (%)
Solution* (non extracted)	5145033	4.5	1133385	N/AP
Plasma	4967384	4.5	1105018	**96.5**
Hemolyzed plasma donor 1	5130093	4.6	1118831	**99.7**
Hemolyzed plasma donor 2	4369303	4.6	943292	**84.9**
Hemolyzed plasma donor 3	4990824	4.5	1114170	**97.0**
Hemolyzed plasma donor 4	4769993	4.4	1085199	**92.7**
Hemolyzed plasma donor 5	5045946	4.5	1130076	**98.1**
Hemolyzed plasma donor 6	4877306	4.4	1110030	**94.8**

*This solution represents a theoreticaly calculated concentrations
**Average from two measurements
***Percent Extraction Yield= Extracted Mean / Non-Extracted Mean x 100

Further analytical method validation evaluations for quantification of phenprocoumon in human plasma were performed successfully according to Algorithme Pharma standard operating procedures (SOP) and according to guidelines from regulatory agencies. The method provide acceptable performance in terms of inter- and intra-batch accuracy and precision **(Table 3).**

Table 3: Summary of (R)-Phemprocoumon Precision and Accuracy

Nominal (R)-Phenprocoumon concentrations (µg/mL)	Plasma QCLOQ 0.50	QC1 1.50	QC2 20.00	QC3 80.00
Intra-batch 1				
Mean concentration (µg/mL)	0.52	1.58	20.81	80.60
S.D.	0.01	0.06	0.51	0.98
Accuracy (% bias)	3.40	5.60	4.00	0.80
Precision (% CV)	2.30	4.00	2.50	1.20
Intra-batch 2				
Mean concentration (µg/mL)	0.51	1.49	20.32	81.16
S.D.	0.02	0.05	0.28	1.96
Accuracy (% bias)	1.00	-0.70	1.60	1.50
Precision (% CV)	3.60	3.10	1.40	2.40
Intra-batch 3				
Mean concentration (µg/mL)	0.50	1.53	20.05	80.03
S.D.	0.01	0.06	0.25	0.71
Accuracy (% bias)	-0.60	2.10	0.30	0.00
Precision (% CV)	2.90	3.90	1.30	0.90

CV: Coefficient of variation; SD: Standard deviation

CONCLUSION

It was demonstrated that an addition of oxalic acid solution to the plasma prior to the liquid liquid extraction effectively improved the recovery (up to 90%) of both (R)- and (S)-phenprocoumon from hemolyzed plasma samples.

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