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Assay of ditiocarb sodium and its methyl ester in mouse plasma by column-switching HPLC

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KEY WORDS ditiocarb; high pressure liquid chromatography; plasma

AIM: To establish a column-switching high pressure liquid chromatograpy (CSHPLC) with direct injection for determination of ditiocarb sodium (DTC) and its methyl ester (DTC-Me) in mouse plasma. **METHODS**: After automated on-line pretreating column enrichment and clear-up, DTC-Me was flushed and chromatographed on an ordinary reversed-phase analytical column, and monitored by UV at $\lambda_{absorption}$ = 276 nm. DTC was methylated before determination. **RESULTS**: No potential interfering peaks were identified. The calibration curves of both analytes were linear within the range of 0.1 - 150 mg·L^{-1}. The correlation coefficients of DTC and DTC-Me were 0.9998 and 0.9995, respectively. The detection limit was 25 μg·L^{-1} and the coefficient of variation in the assay was within 7 % for both compounds. The average recoveries were in the range of 95.28 - 100.06 %. A typical application was presented for mouse after iv DTC 50 mg·kg^{-1}. **CONCLUSION**: The rapid CSHPLC method with direct injection can be used for the study of pharmacokinetics of DTC and DTC-Me.

DTC, a chelating agent, is employed in treatment of nickel poisoning[1]. It was a biological augmenting agent specific for T cells[1,2]. DTC could scavenge the oxygen free radicals and had protective effects on ischemia-reperfusion brain injury[3], and also was active in the opportunistic infections occurring in AIDS patient[4]. Methylation of DTC occurs in the liver and kidneys, resulting in DTC-Me[5]. But its pharmacokinetics was rarely investigated. DTC was labile to acid and heat. The conventional methods for extraction and enrichment of DTC, which was used by some HPLC methods[6-8], are unsuited. To investigate the pharmacokinetics of DTC, a rapid, simple, and sensitive analytic method was needed. This paper reported a column-switching reversed-phase HPLC technique (CSHPLC) for determination of DTC and DTC-Me in mouse plasma.

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MATERIALS AND METHODS

DTC (mp 94 - 96 ℃) was purchased from Sigma. DTC-Me was synthesized by the method of Cobby *et al*[9]. Methanol was of HPLC grade. Water was glass-tridistilled. 2-Mercaptoethanol was purchased from Fluka Chemie. All other reagents were of AR unless indicated.

Stock solution of DTC-Me (500 mg·L^{-1}) was prepared in methanol and kept at -20 ℃. Work solution of DTC was freshly prepared in water just before use.

The chromatographic system employed a Waters Model 510 pump, a Waters Model 490 programmable multi-wavelength detector, a Waters automated gradient controller, a U6K injector equipped with a 200-μL loop, a Model 740 data module (Waters Assoc, Milford MA, USA), and a two-position six-port switching valve (Shanghai Institute of Nuclear Research, Chinese Academy of Sciences) (Fig 1).

The CSHPLC utilized two columns: a 40-μm Bondapak C_{18} (Waters Assoc) pretreating column (PC) (50 mm × 5 mm ID) and a 10-μm YWG C_{18} (Dalian Institute of Chemical Physics, Chinese Academy of Sciences) analytical column (AC) (200 mm × 5 mm ID) for purification and separation, respectively. PC and AC were connected by a switching valve which controlled solvent flow. DTC-Me was retarded selectively on PC, and water-soluble polar coproducts were simultaneously eliminated and eluted into the waste by the pretreating mobile phase ($CH_3OH:H_2O$ - 10:90, vol/vol) at a flow-rate of 3 mL·min^{-1} from pump A. While washing PC for 3 min, AC was equilibrated in the analytical mobile phase ($CH_3OH:H_2O$ - 65:35, vol/vol) with a flow-rate of 1 mL·min^{-1} from pump B. To complete the PC washing cycle, the switching valve was switched from position A to B for another 4 min and was then returned to position A. DTC-Me was flushed by the analytical mobile phase from PC to AC. DTC-Me was separated on AC before determination at 276 nm.

A plasma sample (1 mL) was divided into 2 equal parts, which were used to determine the peak areas of "total DTC"

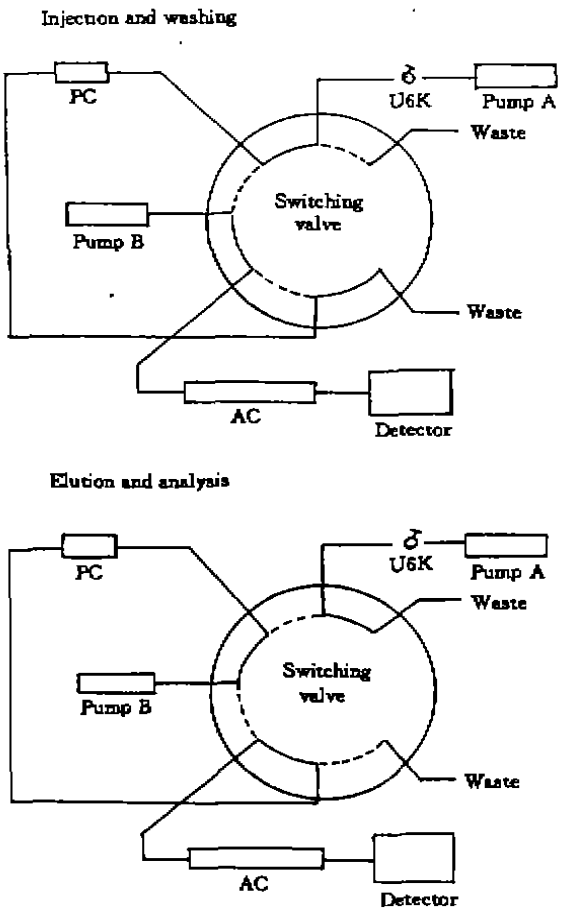

Fig 1. Scheme for a column-switching HPLC system.

(A_T) and DTC-Me (A_M), respectively. One 500-μL plasma sample was methylated as follows: 25-μL phosphate buffer containing edetic acid 0.2 mol · L^{-1} and 2 μL 2-mercaptoethanol were added to plasma. Then methyl iodide (5 μL) was added to the vortexing plasma sample. The plasma was vortexed for additional 30 s and was incubated at 37 ℃ for 30 min prior to dilution with methanol (500 μL). The mixture was centrifuged at 500 × g for 15 min. The supernatant (200 μL) was directly injected into PC for on-line enrichment and purification of the solutes before chromatographic separation on AC. A_T was determined, which consisted of the peak areas of DTC-Me either formated from DTC during methylation procedure or existing in the untreated plasma, or both.

The other 500-μL plasma sample was determined after dilution with methanol as described above but with the omission of the methylation step. A_M was calculated and used to quantitate DTC-Me levels by comparing to its standard curve.

The peak area of DTC (A_D), which was the difference between the peak area of "total DTC" and DTC-Me (A_T-A_M), was used to calculate the concentration of DTC according to its standard curve.

Standard curves were prepared by spiking known amounts of DTC and DTC-Me to fresh heparinized plasma (0.1 – 150 mg · L^{-1}) from drug-free mouse, respectively. The plasma containing DTC-Me was analyzed after dilution with methanol, while that containing DTC was analyzed after methylation. The peak areas of DTC as DTC-Me and DTC-Me were calculated and plotted against their corresponding known concentrations, respectively. Four plasma samples containing 0.5, 2.5, 10, 100 mg · L^{-1} of both DTC and DTC-Me were prepared to test the accuracy and precison of the assay. Another 3 spiked plasma samples containing 10, 20, 40 nmol · L^{-1} of DTC and DTC-Me were prepared to measure the conversion. The samples containing DTC-Me were determined just after dilution, but those containing DTC after methylation. The peak-area ratios of DTC as DTC-Me to DTC-Me were calculated as the % conversion. The detection limits of both DTC and DTC-Me from the analytical mobile phase were defined as a signal-to-noise ratio of 4 : 1. The potential interference from endogenous substances was investigated with blank mouse plasma.

DTC (50 mg · kg^{-1}) was injected to mouse through its tail vein. Heparinized plasma was drawn immediately before injection and was collected through the next 60 min by decapitation. Aliquot (1 mL) of the collection was stored at −20 ℃ until assay.

RESULTS

Chromatography The retention times for DTC as DTC-Me and DTC-Me were all 13.6 ± 0.4 min (n = 5). No interference by the plasma peaks with those of DTC-Me was found. Sample on-line purification, enrichment, and complete separation of DTC-Me were obtained in 15 min (Fig 2).

Calibration curve Standard curves were made by plotting the peak area to spiked concentration (mg · L^{-1}). The curves for DTC and DTC-Me gave good linearities from 0.1 to 150 mg · L^{-1} with correlation coefficients (r) of 0.9998 and 0.9995, respectively.

Validation test The accuracy and reproducibility of the analytical method were investigated by analyzing replicate samples. Coefficients of variation (CV) of both intra- and inter-days were less than 7 % (Tab 1). The recoveries of DTC and DTC-Me from plasma were almost complete (Tab 2).

The conversion of DTC to DTC-Me previously reported at 78 ± 2.4 %[10] was, in our study, found to be 62.8 ± 2.1 % (Tab 3). Both conversion and

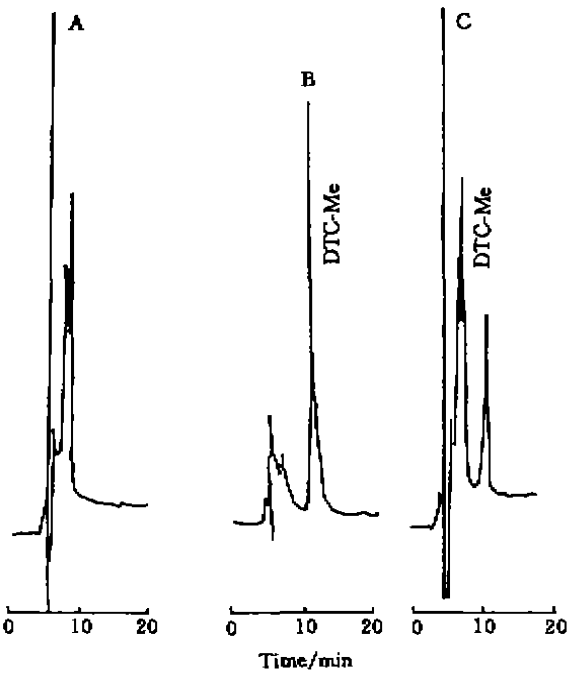

Fig 2. Chromatograms of mouse blank plasma (A), spiked plasma (B), and postdosing 10 min plasma (C) from a mouse iv DTC 50 mg·kg^{-1}. Retention time: 13.6 min.

Tab 1. Coefficient of variation (%) in determination of DTC and DTC-Me in spiked plasma. $n=5$, $\bar{x}\pm s$.

Added/ mg·L^{-1}	Intra-day DTC	Intra-day DTC-Me	Inter-day DTC	Inter-day DTC-Me
0.5	4.26	5.88	6.12	6.25
2.5	3.63	3.57	3.59	3.61
10.0	2.74	1.29	3.03	2.50
100.0	2.76	2.13	2.97	2.54

Tab 2. Recovery of DTC and DTC-Me from spiked plasma. $n=5$, $\bar{x}\pm s$.

Compound	Added/ mg·L^{-1}	Found/ mg·L^{-1}	Recovery/ %
DTC	0.50	0.472±0.013	94.5±1.4
	2.50	2.43±0.04	97.5±1.7
	10.00	10.03±0.20	100.3±2.0
	100.00	100.50±0.92	100.5±0.9
DTC-Me	0.50	0.476±0.018	95.3±4.0
	2.50	2.50±0.06	100.1±2.7
	10.00	9.91±0.16	99.1±1.6
	100.00	100.41±1.33	100.4±1.3

recovery were independent of concentration at the concentration range studied. The detection limits of both compounds from the analytical mobile phase were 25 μg·L^{-1}.

Tab 3. Conversion of DTC to DTC-Me in spiked plasma. A_D/A_M is the ratio of the peak area of DTC (A_D) to that of DTC-Me (A_M) and represents the conversion of DTC to DTC-Me. $n=5$, $\bar{x}\pm s$.

Concentration/ nmol·L^{-1}	A_D	A_M	A_D/A_M
10	639 803±19 833	1 107 975±45 426	61 %
20	1 244 650±46 052	2 015 115±58 438	65 %
40	2 886 417±112 420	4 810 695±23 093	63 %

Applications of the method The plasma concentration of DTC and DTC-Me in mouse after iv DTC 50 mg · kg^{-1} was measured. The pharmacokinetic parameters of DTC and its metabolite DTC-Me were given in Tab 4.

Tab 4. Pharmacokinetic parameters of DTC and DTC-Me in mouse after iv 50 mg·kg^{-1}. $n=5$, $\bar{x}\pm s$.

Parameter Unit	DTC	DTC-Me
$T_{\frac{1}{2}\alpha}$/min	3.0±0.5	
$T_{\frac{1}{2}\beta}$/min	11±3	30±5
$T_{\frac{1}{2}Ka}$/min		4.3±0.8
K_{12}/min^{-1}	0.0085±0.0022	
K_{21}/min^{-1}	0.064±0.012	
K_{10}/min^{-1}	0.22±0.09	
V_b/L·kg^{-1}	1.70±0.25	
Cl_b/L·kg^{-1}·min^{-1}	0.104±0.024	
AUC/min·mg·L^{-1}	480±79	10.3±1.6
T_p/min		14±3
C_{max}/mg·L^{-1}		0.172±0.025

The distribution and elimination of DTC in mouse were rapid. The $T_{\frac{1}{2}\alpha}$ and $T_{\frac{1}{2}\beta}$ were 3.0 and 11 min, respectively. Its metabolite DTC-Me was found in plasma 5 min and attained to peak 10 min after DTC administration.

DISCUSSION

CSHPLC, in which the solid phase extraction was coupled with high pressure liquid chromatography, had been applied to trace enrichment, extraction and analysis of the drugs in biological fluids, and clear up of complex matrices.

After on-line extraction and enrichment by PC, the compounds of interest were separated on AC. This method was highly automated and reduced the time required for sample preparation. Difficult or impossible-to-extract compounds may be assayed by CSHPLC method.

DTC and DTC-Me were labile to acid and heat. The conventional liquid-liquid extraction was unsuitable, so the studies of pharmacokinetics concerned with DTC and DTC-Me were impeded. In the newly developed CSHPLC method, DTC and DTC-Me could be completely and quantitatively analysed directly from plasma without loss and without use of an internal standard as in a conventional sample extraction and preparation procedure. It took only 15 min to extract, enrich, separate, and detect DTC-Me.

Since DTC slowly decomposed in plasma, it was important either to derivatize the plasma quickly or to store the plasma at −20 ℃ until just before derivatization. The methylation of DTC was an extremly critical step in this analysis. Temperature and pH were important factors which affected the percentage conversion of DTC to DTC-Me. To completely reduce all DTC bound to plasma protein-bound cupric ions and to entirely convert it to its methyl ester, it was found necessary to add 2-mercaptoethanol as a reducing agent to the reaction mixture. The successful application of this method demonstrated that it was simple, accurate, and precise enough for the further clinical studies of DTC.

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柱切换高压液相色谱法测定小鼠血浆中二硫卡钠及其甲酯

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关键词 二硫卡酸；高压液相色谱法；血浆

目的：建立一个柱切换 HPLC 法直接进样测定小鼠血中二硫卡钠(DTC)及其甲酯(DTC-Me)浓度. **方法**：血浆(200 μL)经甲醇稀释、离心，取上清(200 μL)进样. 经预柱在线纯化后，切换至分析柱上分离，并用紫外检测 DTC-Me 测定 DTC 血浆水平时，须先用碘甲烷进行甲基化处理. **结果**：血浆内源性物质对测定无干扰，DTC 和 DTC-Me 的线性范围为 0.1－150 $mg \cdot L^{-1}$，其变异系数小于 7 %，最低检测限约为 25 $\mu g \cdot L^{-1}$. 应用该法研究了小鼠 iv DTC 50 $mg \cdot kg^{-1}$ 的药物动力学. **结论**：血浆仅需稀释即可用本法测定，方法快速、简便、准确、灵敏，可用于 DTC 体液水平分析及其药物动力学研究.