

溶剂提取和高效液相色谱-电化学检测法测定人血浆中儿茶酚胺

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Solvent extraction and high performance liquid chromatography with electrochemical detection for determination of plasma catecholamines

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ABSTRACT This paper describes a technique for selectively extracting plasma catecholamines prior to quantification by HPLC-EC. The extraction system was a two-stage process. The first stage involve the complex formation between diphenylborate and catechol (diol) groups in alkaline medium. The second stage was a liquid-liquid extraction. The complex combined with tetraoctylammonium bromide to form an ion-pair formation into organic solvent. The catecholamines in turn was extracted with acid. This technique provided a very specific extraction procedure which resulted in chromatograms with few interfering compounds and gave absolute recoveries (100-103%) of norepinephrine, epinephrine and dopamine. Meanwhile, the plasma catecholamines were concentrated and the detective sensitivity was increased. A good linear relationship was found between the concentrations and ratio of peak heights of the catecholamines from 0.125-2 ng. The correlation coefficients ranged from 0.998-0.999. The coefficients of variation of the intra- and inter-assay were within 3 and 6% respectively. The results show that the procedure is very simple and fast. The method is valuable not only for clinical diagnosis but also for laboratorial research.

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KEY WORDS high pressure liquid chromatography; electrochemistry; catecholamines; plasma

摘要 本文报道用溶剂提取系统对生物样本进行预处理, 然后用高效液相色谱-电化学检测测定人血浆中儿茶酚胺, 此法对生物样本的预处理简单、快速, 能专一地提取血浆中的儿茶酚胺, 有效地除去杂峰的干扰, 同时使血浆中的儿茶酚胺浓缩, 减少取样量, 提高测定的灵敏度。在 0.125-2 ng 之间去甲肾上腺素和肾上腺素的浓度同峰高比之间有良好的线性关系, 绝对回收率可达 95% 以上。

关键词: 高压液相色谱法; 电化学; 儿茶酚胺; 血浆

儿茶酚胺在血浆中含量极低, 并存在其它一些具有电化学活性物质的干扰。因此选择有效的血浆预处理方法, 是成功地运用高效液相色谱法测定血浆中儿茶酚胺含量的关键。近年来国内外都已报道⁽¹⁻³⁾用氧化铝吸附法提取血浆中儿茶酚胺, 但此法对儿茶酚胺提取的专一性不强, 回收率较低。本文采用一种溶剂提取系统, 对血浆进行预处理, 然后用反相高效液相离子对色谱-电化学检测法(HPLC-EC)测定人血浆中儿茶酚胺的含量。实验证明此法操作简便快速, 回收率高, 对儿茶酚胺提取的专一性强。用本法测定人和大鼠尿儿茶酚胺的含量也已获得成功(未发表资料)。

MATERIALS AND METHODS

仪器 Beckman HPLC 系统, 其中包括

110 B 型输液泵, ALTEX 210 A 进样阀, BAS LC-4 B/17 型电化学检测器(Bioanalytical System Inc, 玻璃碳检测电极, Ag/AgCl 参比电极), 472 数字处理器。

试剂 去甲肾上腺素(norepinephrine NE, Serva); 肾上腺素(epinephrine E) 和多巴胺(dopamine DA)为 Fluka 产品; 3, 4-二羟基苄胺(dihydroxybenzylamine DHBA, Sigma)。二苯基硼酸 2-氨基乙酯(diphenylboric acid 2-amino ethyl ester DPBEA, Sigma), 四辛基溴化铵(tetraoctylammonium bromide TOABr, Fluka), 离子对色谱试剂(IPR-B₇), 天津化学试剂二厂), 以及正庚烷、正辛醇和乙二胺四乙酸(EDTA) 等其它国产 AR 或 CP 级试剂, 水为超纯水(本院中心测试室)。

色谱条件 色谱柱为 Ultrasphere ODS, 250 × 4.6 mm ID, 颗粒度 5 μm; 流动相为乙酸-乙酸钠缓冲液(pH 4.0, 含有 0.68% NaAC, 0.01% EDTA, 1% 离子对色谱试剂, 5% 乙腈使用前以 0.45 μm 滤膜过滤并脱气, 流量为 1.0 ml/min, 电化学检测工作电压 650 mV, 检测灵敏度为 5 nA。

标准溶液配制 将标准品 NE, E, DA 和 DHBA(内标)分别溶于 HClO₄(0.1 mol/L), 配成 0.1 mg/ml 浓度的储存液, 置 4% 避光保存, 1—2 月更换一次。使用时用乙酸(0.08 mol/L)稀释到所需浓度, 进样量为 100 μl。

血样制备 取新鲜 EDTA 抗凝血, 离心(2000 × g/10 min, 4 °C), 分离出血浆, -70 °C 储存备用。测定时取血浆 1.5 ml 具塞试管内, 加入一定量的 DHBA, 1 ml NH₄Cl-NH₄OH 缓冲液(2.0 mol/L, pH 8.5, 含有 0.2% DPBEA 和 0.5% EDTA), 5 ml 正庚烷(含有 1% 正辛醇和 0.1% TOABr)混旋 30 s, 离心(2000 × g/10 min, 4 °C)吸取 4 ml 有机相于另一具塞试管内, 加入 2 ml 正辛醇, 200 μl 乙酸(0.08 mol/L), 混旋 30 s, 离心(2000 × g/5 min, 4 °C)弃去有机相, 取 100 μl 乙酸液注入色谱系统分析。整个提取过程在 30 min 内

即可完成。

含量计算 采用内标法计算。以血浆中 NE 含量的计算为例。将相同浓度的 NE 与 DHBA 的标准品混合液和血浆样本(内加等量的 DHBA)在相同条件下提取后进样。先求出标准品管中 DHBA 和 NE 的峰高比作为响应因子 P_f , ($R_f = H_{DHBA}/H_{NE}$)。血浆样本管 NE 的含量按公式 $C_{NE} = H_{NE}/H_{DHBA} \times C_{DHBA} \times R_f$ 进行计算。其中 C 为浓度, H 为峰高。

RESULTS AND DISCUSSION

溶剂提取法的原理及特点 DPBEA 的硼酸根离子在碱性条件下能与儿茶酚胺中的儿茶酚基团结合, 并带有负电荷, 此复合物再与带有正电荷的 TOABr 结合成离子对的形式转入有机相, 最后用酸性溶液提取, 使儿茶酚胺与酸形成可溶性盐转入水相中⁽⁴⁾。实验证明, 在提取过程中如果不加 DPBEA 和 TOABr, 儿茶酚胺则完全不能提出。此法能选择性地提取含有儿茶酚结构的化合物, 能有效地除去血浆中杂质的干扰, 同时在提取过程中使血浆的儿茶酚胺进一步浓缩, 提高了测定的灵敏度。

分离条件的选择 本文观察了流动相的 pH 值, 离子对试剂的浓度和有机溶剂改性剂对样本分离的影响, 以及 EC 检测器工作电极

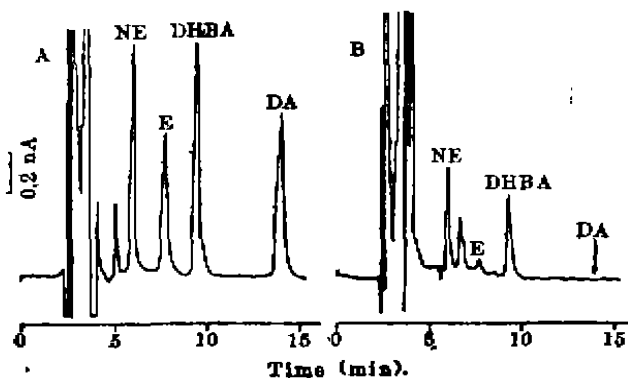


Fig 1. Chromatograms of catecholamines in 100 μl of standard solution 1 ng (A) or of the extracts from human plasma (B) NE = norepinephrine, E = epinephrine, DHBA = 3, 4-dihydroxybenzylamine, DA = dopamine.

Tab 1. Reproducibility of retention time and ratio of peak height, each component 2 ng in 100 μ l standard solution. $n=5$, $\bar{x}\pm SD$.

	Retention time(min)	CV%	Ratio of peak height	CV%
EN	4.546 \pm 0.021	0.46	0.854 \pm 0.006	0.67
E	5.662 \pm 0.019	0.34	0.625 \pm 0.004	0.61
DHBA	6.762 \pm 0.017	0.25	—	—
DA	9.666 \pm 0.009	0.09	0.545 \pm 0.006	1.12

电位的选择。发现在 pH 4.0, 离子对试剂浓度 1%, 乙腈含量为 5%, 以及工作电压在 650 mV 时能使 NE, E 和 DA 获得完全的分离, 血浆样本的分离效果也较好(Fig 1)。标准品进样量在 0.125-2 ng 的范围内与峰高比之间呈良好的线性关系, NE, E 和 DA 的相关系数分别为 0.998, 0.999 和 0.999。并计算了标准品重复进样的保留时间以及 NE, E 和 DA 与 DHBA 峰高比的变异系数(Tab 1)。结果表明标准差和变异系数均较小, 说明方法的重现性较好, 完全适用于微量儿茶酚胺的定量分析。

回收率及精密度 先配制一定浓度系列的标准溶液直接进样, 再将这一浓度系列的标准液分别与 1.5 ml 同一混合血浆混合, 按样本制备方法进行提取分离后再进样, 绘出峰高对浓度的曲线图(Fig 2), 分别计算两条曲线的斜率。回收率按公式 $K_1/K_2 \times 100\%$ 计算, 其中 K_1 为血浆加标准品经溶剂提取后所得曲线的斜率, K_2 为标准品直接进样所得曲线的斜率。

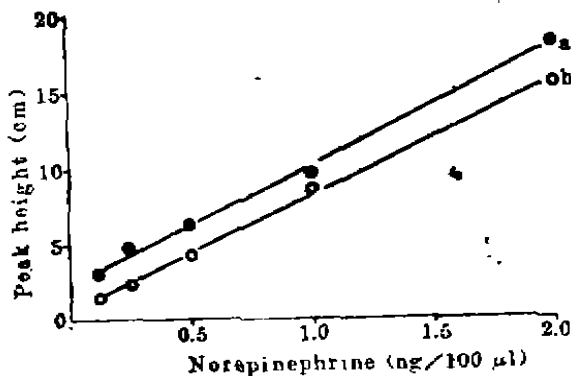


Fig 2. Linearity of response for norepinephrine in spiked plasma with solvent extraction (a) and in NE standard representing 100% recovery (b).

Tab 2. Recovery and precision of determination of NE, E and DA in plasma by this method. $n=5$.

	Recovery (%)	Within-run CV (%)	Between-run CV (%)
NE	102.7	1.6	3.8
E	101.7	2.2	4.0
DA	100.6	1.3	5.9

结果表明, 与氧化铝提取法比较溶剂提取法具有较高的回收率。将一定量的标准品加入混合血浆, 重复提取后测定批内和批间的变异系数(CV), 三项指标的 CV 分别小于 3 和 6% (Tab 2)。

正常人血浆儿茶酚胺含量的测定 用本法测定了二组共 20 例正常人血浆儿茶酚胺的含量, 结果表明两组间 E 的含量非常接近, NE 的含量稍有差别。正常人的年龄、情绪⁽²⁾、活动状态⁽⁵⁾ 都会影响测定结果, 这种差别可能是正常人的个体差异引起。血浆中儿茶酚胺含量的测定国内外均有报道, 但由于取样不同或实验方法不同, 所得的结果差异较大。我们实验的结果与文献值^(3,4,6,7) 比较接近中间水平 (Tab 3)。本法未能测出血浆具 DA 的含量, 这与国内外大部分文献报道的结果^(3,4,6,7) 相符。人血浆中 DA 主要以结合形式存在⁽⁸⁾, 95% 以上正常人血浆中游离的 DA 不能被检出⁽⁹⁾。因此如何用 HPLC-EC 法测定血浆中结合形式或总的儿茶酚胺的含量, 也是一个值得

Tab 3. Results of determination of catecholamines in human plasma by present method compared with values stated in literature. $\bar{x}\pm SD$ (pg/ml).

n	NE	E	Ref
10	521 \pm 77	119 \pm 37	This work
10	407 \pm 55	116 \pm 34	
10	194 \pm 34	200 \pm 49	(3)
1	399	70	(4)
1	515	125	(6)
1	357	74	(7)
1	415	201	*

*Related BAS publications. Tissue catecholamines. LCEC Application Note No 12

研究的课题。从血浆的色谱图看在 NE 和 E 之间有一未知峰, 20 例正常人血浆中均有这一峰出现, 对此峰的定性还有待研究。

本法测定简便、快速, 不仅能测定血浆中儿茶酚胺的含量, 也能测定尿中儿茶酚胺的含量, 提示本法对临床诊断和实验室研究有一定的应用价值。

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